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Germline mutations in *BRCA1* lead to an increased risk of breast and ovarian cancer, with loss of the second, normal allele critical to tumorigenesis. It was widely presumed that the cloning and characterization of genes involved in hereditary breast cancer would lead to a better understanding of the genesis of the more common non-inherited forms of breast cancer. In several ways, *BRCA1* fits the classic tumor suppressor model. Still, the relative lack of somatic mutations found in *BRCA1* has argued against its involvement in non-inherited breast cancer.

Our research specifically addresses whether transcript-specific regulation of *BRCA1*, CpG methylation, or large genomic rearrangements are responsible for somatic inactivation of *BRCA1*. As neither transcript-specific regulation nor CpG methylation appeared to explain the lack of *BRCA1* somatic mutations, we have chosen to focus on whether *BRCA1* is inactivated somatically by large genomic rearrangement. Despite the fact that our series of breast tumor/normal tissues is typical in the degree of LOH detected within the *BRCA1* region, no large rearrangements have been detected using long range PCR. It is premature to draw conclusions as we have not finished Southern analysis in this group of tumors. Nonetheless, if no large somatic rearrangements are detected using both long range PCR and Southern analysis, then we must conclude that *BRCA1* is not likely to be inactivated by mutation in sporadic breast tumors.

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Joanne L. Payne 11·11·98

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Table of Contents
Predoctoral Fellowship Annual Report 1998
Shannon R. Payne
Grant DAMD17-97-1-7210

Introduction

In 1990, a breast cancer susceptibility gene, *BRCA1*, was mapped to chromosome 17q21 (1). Germline mutations in *BRCA1* lead to an increased risk of breast and ovarian cancer, with loss of the second, normal allele critical to tumorigenesis. The human *BRCA1* gene encodes a 1863 amino acid polypeptide whose biochemical function is still unclear, although proteins interacting with *BRCA1* genetically and/or physically have been identified (2,3,4).

Most germline *BRCA1* mutations are small insertions, deletions or single bp substitutions which lead to premature protein truncation (5). These mutation classes are rarely found as somatic mutations in *BRCA1*. Conversely, somatic deletions of multiple contiguous megabases (Mb) including *BRCA1*, as reflected by loss of heterozygosity (LOH), occur frequently in both inherited and non-inherited breast and ovarian cancer (6).

It was widely presumed, based on the classic tumor supressor genes *TP53* and *RB1*, that the cloning and characterization of genes involved in hereditary breast and ovarian cancer would lead to a better understanding of the genesis of the more common non-inherited (sporadic) forms of breast and ovarian cancer. In several ways, *BRCA1* fits the classic tumor suppressor model: 1) expression of *BRCA1* decreases during sporadic breast cancer progression (7) 2) inhibition of *BRCA1* accelerates mammary epithelial cell growth (7) and 3) LOH including the *BRCA1* region occurs frequently in sporadic breast cancer (6). Still, the relative lack of somatic mutations found in *BRCA1* has argued against its involvement in sporadic breast cancer.

The research described within this report seeks to answer whether and how *BRCA1* is somatically inactivated in sporadic breast cancer. Since *BRCA1* mRNA has been seen to decrease during sporadic breast cancer progression, transcriptional deregulation of *BRCA1* is a plausible explanation for the lack of somatic *BRCA1* mutations in sporadic breast cancer. For this reason, our original proposal contained the following objectives:

1. Precisely define the transcripts of *BRCA1* and *1A1.3B* (a closely linked gene).
2. Quantitate the levels of the distinct *1A1.3B* and *BRCA1* transcripts in breast cell lines.
3. Determine whether *1A1.3B* and *BRCA1* are regulated in tandem.
4. Determine whether transcript-specific regulation is disrupted in breast cancer.
5. Determine the functional elements of the BRCA1 promoter region.

In the time since our proposal was originally submitted, a number of groups have been involved in elucidating and publishing the promoter structure of *BRCA1*. The

genomic structure of the putative promoter region of *BRCA1* revealed that *BRCA1* lies within a duplicated region of chromosome 17 (8,9). Additionally, *BRCA1* does not share a promoter with *1A1.3B* (now known as *NBR1*). Rather, it shares a promoter with a gene likely created by the duplication event, known as *NBR2*.

BRCA1 contains two distinct transcription start sites leading to alternate exons 1 (1a and 1b) which result in two different 5' UTR's (10). Xu and colleagues (11) also determined that *BRCA1* is transcriptionally regulated by two distinct promoters (α and β) generating the two distinct transcripts identified previously. Promoter α is a bi-directional promoter shared with *NBR2* which results in a transcript containing exon 1a. Both promoter α and promoter β are responsive to estrogen. Additionally, there have been two reports of a putative negative regulatory element within the *BRCA1* promoter α (11,12). The two reports disagreed, however, on the location of the "silencer" element. Xu and colleagues (11) placed the "silencer" within a 167 basepair (bp) fragment at -223 to -389 bp upstream of *BRCA1* exon 1a. Rice and colleagues (12) placed it within a 391 bp fragment at -567 to -957 bp upstream of *BRCA1* exon 1a.

The complete genomic structure of *BRCA1* revealed that both promoter α and promoter β lie within a CpG island (13). Aberrant cytosine methylation at CpG dinucleotides can lead to transcriptional repression. A number of tumor supressor genes, e.g. *RB1, CDKN2, hMLH1*, are abnormally methylated in tumor cells (14,15,16). This aberrant methylation has been correlated with transcriptional repression of the methylated allele. Thus, abnormal methylation at CpG dinucleotides within the *BRCA1* CpG island is one potential mechanism for somatic inactivation of *BRCA1*.

Clearly, *BRCA1* somatic mutations detectable by conventional PCR-based techniques are extremely rare in breast tumors. These conventional screening procedures, however, neglect another class of mutations: large genomic rearrangements. Both inherited and somatic large genomic rearrangements are a common feature of many human diseases including muscular dystrophy (17), Charcot-Marie-Tooth disease (and the mechanistically related hereditary neuropathy with liability to pressure palsies) (18), and familial hypercholesterolemia (19). Several large germline deletions in *BRCA1* have been reported since the original submission of our proposal (Table 1), all of which were undetectable by conventional genomic DNA screening procedures. Large germline deletions appear to be a relatively common feature of inherited breast cancer in the Dutch population, due in large part to founder mutations (20). It is still unknown whether large genomic rearrangements might contribute to somatic inactivation of *BRCA1* in sporadic breast tumors. Several

features of the *BRCA1* locus provide clues as to the nature of large genomic rearrangements which might occur.

BRCA1 has one of the highest densities of Alu elements of genes deposited in GenBank (13). Alu elements have been implicated in many human disease-associated gene rearrangements including familial hypercholesterolemia (21), hypobetalipoproteinemia (22), hereditary angioedema (23), *ADA*$^{-}$ severe combined immune deficiency (24), and Ehlers-Danlos Syndrome Type IV (25). In most cases for which *BRCA1* breakpoint junctions were characterized, at least one deletion breakpoint occurred within Alu sequence (20,26,27). Large genomic rearrangements in Alu-dense genes do not necessarily entail a high frequency of breakpoints within Alu elements (28). We have characterized a large germline deletion in the *BRCA1* region which does not contain a breakpoint in Alu sequence and appears to have occurred by a non-homologous mechanism (see Body). To date, however, most characterized large germline deletion breakpoints in *BRCA1* have occurred in Alu sequence.

Another predisposing feature of the *BRCA1* locus is the tandem duplication involving the 5' region of *BRCA1* (8,9). The duplication includes the *BRCA1* promoter region and a neighboring gene, *NBR1*. The result of the duplication was the creation of a new gene, *NBR2*, with which human *BRCA1* shares a bi-directional promoter (11) and the creation of a pseudo-copy of *BRCA1* situated in a similar head to head orientation with the *NBR1* gene approximately 30 kb upstream of the functional *BRCA1* locus. The pseudo-copy of *BRCA1* contains a nonsense mutation in exon 2 (8). The presence of a pseudo-copy of *BRCA1* with >90% identity located so near on chromosome 17 further predisposes the functional *BRCA1* locus to inactivation by misaligned homologous recombination or gene conversion.

Our research specifically addresses whether transcript-specific regulation of *BRCA1*, CpG methylation, or large genomic rearrangements are responsible for somatic inactivation of *BRCA1*. As neither transcript-specific regulation nor CpG methylation appeared to explain the lack of *BRCA1* somatic mutations, we have chosen to focus on whether *BRCA1* is inactivated somatically by large genomic rearrangement. Our large sample size (93 paired tumor/normal samples to date) combined with the dual screening approach of long range PCR and Southern analysis will permit a clear answer with respect to this question. If large genomic rearrangements in *BRCA1* do occur in sporadic breast tumors, then we can begin to formulate questions as to when, how and why *BRCA1* is targeted. If large rearrangements in *BRCA1* do not occur in sporadic breast tumors, then

we can more vigorously investigate alternative mechanisms of inactivation such as loss of as yet unidentified upstream regulatory factors.

Body

Transcript-specific regulation:

Our stated goal for this research was to study the transcriptional regulation of *BRCA1* as a tool for understanding whether and how BRCA1 might be involved in sporadic breast cancer. As a preliminary experiment to gain qualitative insight into the transcription profile for the two distinct transcription start sites of *BRCA1*, we designed transcript-specific primers and performed RT-PCR on a panel of breast, ovary, and prostate cell lines (including normal human mammary epithelial cells). RT-PCR products for both *BRCA1* exon 1a and exon 1b transcripts were detected in all cell lines examined.

In order to determine whether quantitative differences were present in the levels of transcript α and transcript β between cell lines, we performed Rnase protection assays using probes specific to the distinct exons 1 of each transcript. Although control probes (such as *GAPDH,* Cyclophilin, β-actin) routinely worked well, we encountered difficulty in attempting to detect *BRCA1* mRNA with exons 1a/1b-specific probes. This difficulty could be due to a number of factors. First, *BRCA1* transcripts are generally detected at a low level. Attempting to further subdivide this pool to detect only a single species of *BRCA1* transcript is likely to be technically difficult. Second, exon 1b contains a fragment of an Alu repetitive element which complicates the generation of a specific probe for transcript β. Finally, the probes used to distinguish transcripts α and β were necessarily located within the 5' UTR of *BRCA1*. It is well-established that 5' UTR's often assume secondary structure that may interfere with binding to a complementary strand probe.

CpG Methylation:

Because the structure of the *BRCA1* promoter was described shortly after this research proposal was submitted, we focused our research efforts on determining whether abnormal methylation of the *BRCA1* CpG island leads to transcriptional repression. We planned to use a combination of methylation-specific PCR, sodium bisulfite genomic sequencing and Rnase protection assays (not transcript-specific) to address this question.

We first looked at cytosine methylation in a number of cell lines using methylation-specific PCR. In this technique, a small amount of genomic DNA is digested with a methylation sensitive enzyme and then subjected to PCR amplification around the digested

region. Only those samples which contain a methylated cytosine to protect them from digestion are successfully amplified. In this manner, we identified cytosine methylation in a number of cell lines of both normal and tumor tissue origin. In general, normal human mammary epithelial cells exhibited the least degree of methylation whereas an epidermoid tumor line and lymphoblast line exhibited the most widespread methylation. Still, we could observe no correlation between the degree or specific sites of methylation and the mRNA expression level of *BRCA1* in these cell lines.

There have been two reports of aberrant cytosine methylation of the *BRCA1* promoter in breast tumors (29,30). In one study, two of seven and in the other study, two of six sporadic breast tumors contained extensive cytosine methylation. Neither of these studies, however, correlated methylation with expression of *BRCA1*. More recently, Rice and colleagues (12) examined 7 breast cancer cell lines using sodium bisulfite genomic sequencing and RT-PCR. Although the level of *BRCA1* mRNA was decreased compared to normal human mammary epithelial cells in all the breast cancer cell lines examined, only one breast cancer cell line showed extensive CpG methylation. Whether this is a true correlation between CpG methylation and expression level is unclear from the data.

Large genomic rearrangement of BRCA1:

As neither transcript-specific regulation nor CpG methylation appeared to offer sufficient explanation for the lack of somatic mutations in *BRCA1*, we considered another explanation. Given the genomic organization of the *BRCA1* locus and the methods commonly used to screen for *BRCA1* mutations, it is conceivable that *BRCA1* somatic mutations in breast cancer exist but have remained undetected by conventional mutation detection methods.

We have studied this possibility using a combination of long range PCR and Southern analysis. Fifteen unique long range PCR primers (Table 2) were designed across the *BRCA1* genomic region. The pairs amplify overlapping products ranging in size from 2.4 to 10.9 kilobases (kb) with an average size of 7.7 kb. The primer pairs provide full coverage of the *BRCA1* region, from the promoter to 5 kb downstream of the final exon with one exception. The region (~24,500-28,700 of GenBank L78833) encompassing exon 6, exon 7 and the Alu-dense intron 7 was refractory to long range PCR amplification. A primer pair amplifying a smaller 3.8 kb product was designed to screen exons 6 and 7. However, a 1187 bp gap in intron 7 remains. This region will be screened solely by Southern analysis.

Long range PCR with these primer pairs was used to screen for large genomic rearrangements in the *BRCA1* region. After amplification, the long range PCR products

were separated in 0.8% agarose and analyzed for gross size differences. The long range PCR products were also subjected to restriction enzyme digestion and analyzed for variant banding patterns. We began by investigating a series of 11 high risk breast cancer families in which no *BRCA1* mutations had been identified using conventional mutation detection methods. Four of these families demonstrated clear linkage to *BRCA1*. In the other seven families, too few affected individuals were available for informative linkage analysis. The families were screened using lymphocyte genomic DNA from one linked and one unlinked family member. In the event that linkage was unclear, two high risk individuals (when available) were screened. In parallel, a series of 6 breast (BT20, HBL100, MDA-MB-157, MCF7, MDA-MB-231, T47D), 2 ovarian (ES2, PA1), and 3 prostate (DU145, PC3, PPC1) cancer cell lines were examined.

To address whether large rearrangements occur as somatic mutations in BRCA1, unselected paired tumor/normal samples were screened by long range PCR followed by restriction enzyme digestion. The samples were obtained through collaboration with two separate groups: the Cooperative Human Tissue Network (CHTN) and the Louisiana Women's Hospital in Baton Rouge. CHTN is a NIH-supported resource which distributes tissue samples to labs across the United States for research. The 45 samples from this group are analyzed with respect to such parameters as estrogen and progesterone receptor status. The Louisiana Women's Hospital has graciously agreed to send samples specifically for BRCA1 research. These 48 samples come with a detailed family history for each patient. Samples from both groups are obtained on a prospective basis.

Results of Long range PCR Analysis:

Analysis of one family (Family 5) revealed germline deletion of exon 3 in the mRNA which cosegregated with 10 cases of breast and ovarian cancer and led to premature protein truncation at codon 27 (31). SSCP analysis of cDNA indicated that *BRCA1*-linked members of Family 5 were heterozygous for a *BRCA1* transcript lacking exon 3 (Fig.1A). No other alterations were detected in genomic or cDNA. Splice junctions of exons 2, 3 and 5 were sequenced from PCR products using primers immediately flanking the exons, but no mutations were observed (31). As only RNA from lymphoblasts was available, it was unclear whether the transcript lacking exon 3 was merely a rare variant observed in lymphoblasts or a true disease-causing mutation. The genomic basis of the mRNA deletion remained unknown.

Using a primer pair designed to amplify a 5531 bp product encompassing exon 3, a variant long range PCR product was detected in members of Family 5 (Fig.1B). The smaller product segregated with the *BRCA1*-linked haplotype in the family. All family

members heterozygous for transcripts lacking exon 3 were also heterozygous for the variant long range PCR product.

Wild-type and variant long range PCR products were cloned and sequenced from multiple family members. Sequence of all variant clones from two different family members consistently revealed the complex mutation shown in Figure 2: a 10 bp inverted duplication followed by a 1039 bp deletion in *BRCA1* exon 3 and intron 3. In the mutant sequence, bp 1-22 of exon 3 are wild-type. At position 23 of exon 3, a 10 bp inversion of bp 11-20 is inserted. The nucleotide after the insertion is bp +1008 of intron 3. The net deletion is 1029 bp, yielding an amplified product of 4502 bp which is consistent with the electrophoretic mobility of the variant long range PCR product. No other rearrangements of *BRCA1* were detected in Family 5 using the long range PCR screen.

No other large genomic rearrangements were detected in the families or the cell lines using long range PCR. Additionally, no large genomic rearrangements were observed in any of the 84 paired tumor/normal breast tissue samples fully screened by long range PCR. Several restriction fragment length polymorphisms (RFLP) were observed. The RFLP's identified thus far by this screen are listed in Table 3.

Loss of Heterozygosity within the *BRCA1* Region:

Because no large genomic rearrangements were observed in the paired tumor/normal breast tissue samples, loss of heterozygosity (LOH) analysis was performed on these samples prior to Southern analysis. Consequently, only those samples exhibiting *BRCA1* LOH (and thus, most likely to contain a *BRCA1* mutation) would be subjected to Southern analysis.

LOH in tumor samples was assessed by using highly polymorphic markers in the *BRCA1* region (Table 4). The markers are typically dinucleotide repeats of variable length and were amplified using published primer pairs. The PCR products were radioactively labelled and then separated by polyacrylamide gel electrophoresis. Six markers in the *BRCA1* region were used: one proximal marker (D17S1320), all three intragenic markers (D17S855, D17S1322, D17S1323), and two distal markers (D17S1326, D17S1325). Of the 92 paired tumor/normal breast tissue samples successfully amplified for all six markers, 42 breast tumor samples demonstrated LOH for at least one marker (45%). Of 31 tumor/normal pairs informative for all three intragenic *BRCA1* markers, 15 tumors demonstrated LOH. An additional 22 tumors which were not informative for all three intragenic markers demonstrated LOH at one or more of the *BRCA1* intragenic markers.

Southern Analysis of *BRCA1*:

Southern analysis of the *BRCA1* region proved technically more difficult than long range PCR and LOH analysis. This was likely due to the single copy nature of *BRCA1*, a large gene with a number of small exons which are spread over a large genomic region (81 kb). Problems with specificity of the probe fragment, Southern transfer methods, and sensitivity were encountered. Eventually, three suitable cDNA probe fragments were identified. These are as follows: C1F/C2R (exons 1a-7), C2F/C4R (exons 7-11), and C5F/C11R (exons 11-24). Primer sequences are as described in (31). Efficiency of Southern transfer was improved by eliminating the depurination step and substituting a brief UV nicking step. Greater sensitivity was achieved by performing the hybridization in formamide at 42° and extending the incubation to 3 days.

We are currently screening the family and cell line DNA by Southern analysis. To date, no further large rearrangements have been detected. Next, we will screen the 42 breast tumor samples which demonstrated LOH within the *BRCA1* region.

Conclusions

In summary, we began this project with the stated purpose of studying the transcriptional regulation of *BRCA1* as a tool for understanding whether and how *BRCA1* might be involved in sporadic breast cancer. As preliminary experiments, we investigated whether the alternative transcription start sites might confer tissue-specificity for the two distinct *BRCA1* trancripts. We did not, however, observe differential expression of the two transcripts. Because the complex regulation of the *BRCA1* promoter was described shortly after this work began, we chose to focus instead on whether *BRCA1* might be inactivated in sporadic breast tumors by CpG methylation. There have been a few reports of CpG methylation at the *BRCA1* promoter region in breast tumors. These reports did not, however, examine *BRCA1* mRNA expression levels in these tumors. In our lab, we have thus far observed no correlation between methylation and transcriptional repression of *BRCA1*.

Several possible interpretations could explain the paradox presented by the lack of somatic mutations in *BRCA1*. One formal explanation is that *BRCA1* is not involved in sporadic breast cancer. It is possible that the high rate of LOH for the *BRCA1* region in sporadic tumors actually targets a nearby gene, and not *BRCA1*. A second possibility is an alternative mechanism for loss of the second *BRCA1* allele in sporadic tumors. For example, *BRCA1* expression might be suppressed by CpG methylation or the loss of a necessary transcription factor upstream of *BRCA1*. A third explanation is that the lack of

BRCA1 mutations in sporadic tumors actually reflects limitations in mutation screening procedures.

With little evidence to support the hypothesis that *BRCA1* is inactivated somatically by CpG methylation and little known about the transcription factors required for *BRCA1* expression, we began to investigate whether the apparent lack of somatic mutations in *BRCA1* might actually reflect limitations in mutation screening procedures.

It is clear that large genomic rearrangements do occur as germline mutations in *BRCA1* (20,26,27). Of primary importance is whether such mutations occur somatically as well as in the germline. Breast cancer is the most common malignancy among women in North America and Northern Europe (32,33). However, only 5-10% of all breast and ovarian cancer is attributable to inherited mutations (34). Several lines of evidence support a role for BRCA1 as a tumor suppressor in sporadic breast cancer. First, antisense inhibition of *BRCA1* accelerates the growth of mammary epithelial cells in tissue culture (7). Additionally, expression of *BRCA1* decreases during sporadic breast cancer (7). Finally, as mentioned, LOH of the *BRCA1* region occurs frequently in sporadic breast cancer. Despite these observations, *BRCA1* mutations have not yet been detected in sporadic breast tumors (35). One explanation is that the spectrum of somatic mutation in *BRCA1* differs dramatically from the germline mutation spectrum.

We have used long range PCR and Southern analysis to identify large genomic rearrangements (both germline and somatic) within the *BRCA1* region. We began by screening a series of 11 high-risk breast cancer families in which no *BRCA1* or *BRCA2* mutations had been detected using conventional screening procedures. We identified one such germline mutation in Family 5, a family with demonstrated linkage to the *BRCA1* region. The mutation in Family 5 arose from a 1039 bp deletion and results in exon skipping of exon 3, leading to a frameshift and premature protein truncation. The mutation in Family 5 is notable in that unlike previously described large germline deletions in *BRCA1*, neither breakpoint resides within an Alu element.

Despite the fact that our series of breast tumor/normal tissues is typical in the degree of LOH detected within the *BRCA1* region, no large rearrangements were detected using long range PCR. It is premature to draw conclusions as we have not finished Southern analysis in this group of tumors. Nonetheless, if no large somatic rearrangements are detected using both long range PCR and Southern analysis, then we must conclude that *BRCA1* is not likely to be inactivated by mutation in sporadic breast tumors. Remaining alternatives include the preferential loss of an upstream transcription activator for *BRCA1* or the presence of a closely linked tumor supressor on chromosome 17q21.

Table 1:

Previously Reported
Large Germline Deletions in *BRCA1*

Location	Size	Features	Effect	Ref.
ivs 16 to ivs 17	1008 bp	breakpoints in 2 oppositely oriented Alu elements majority of both Alu elements removed	exon 17 deleted	26
NBR2 to ivs 2	14 kb	breakpoints in right arm of 2 Alu elements consensus Alu element reconstituted	no transcript	27
ivs 12 to ivs 13	3835 bp	3' of ivs 12 Alu joined to region of high homology w/ ivs 12 Alu Dutch founder mutation	exon 13 deleted	20
ivs 21 to ivs 22	510 bp	breakpoints in ivs 21 and most 5' copy of three head-to-tail ivs 22 Alu elements; Dutch founder mutation	exon 21 deleted	20
exon 3 to ivs 3	1039 bp	breakpoints in exon 3 and ivs 3; non-homologous mechanism insertion of 10 bp inverted duplication at junction	exon 3 deleted	submitted

Table 2:

Long Range PCR Primer Pairs

Pair No.	*Primer Name*	*Annealing Temp.*	*Size (bp)*	*GenBank L78833*	*Sequence*
1	n2.1.1bR	65	2364	2309-4672	5'-GCA ATG CAA AGA CCG TCC GCT G-3'
	BA2				5'-GTA CTT CTT CAA CGC GAA GAG CAG ATA AAT C-3'
2	1aF	65	9936	3028-12963	5'-TTT GGA CAA TAG GTA GCG ATT CTG ACC TTC-3'
	3R				5'-AAC TCC AGA CTA GCA GGG TAG GGG GGG-3'
3	3F	61	5531	12792-18322	5'-TCC TGA CAC AGC AGA CAT TTA-3'
	4R				5'-CCC GTC TCT ACA GAA AAC AC-3'
4	ivs3F	65	10857	13715-24571	5'-TGT GAA GAC AGG AAA GGA CCT GAT ACC AGT TTC-3'
	7R				5'-CAC GGT TTC TGT AGC CCA TAC TTT GGA TGA TAG-3'
5	6F-2	65	3828	23839-27666	5'-GCT TTT CAG CTT GAC ACA GGT TTG G-3'
	ivs7R				5'-CCC CAG CAC TCC TAA GAA CAT TTA GTA TAG G-3'
6	8F	65	8361	28853-37213	5'-CAG GAA ACC AGT CTC AGT GTC CAA CTC TCT AAC CTT G
	11R				5'-TGT CAC TCA GAC CAA CTC CCT GGC TTT CAG AC-3'
7	11F	65	9518	36783-46300	5'-CCA TAC ACA TTT GGC TCA GGG TTA CCG AAG AGG G-3'
	13R				5'-TTC GCA GGT CCT CAA GGG CAG AAG AGT CAC-3'
8	12F	65	8927	37715-46641	5'-GGT GTG AGA GTG AAA CAA GCG TCT CTG AAG ACT GC-3
	ivs13R				5'-GCC TGT CAC CAA TTT CTC CCA TTC CAC TTA GCT TC-3'
9	13F	65	8071	46235-54275	5'-GGA GCC AGC CTT CTA ACA GCT ACC CTT CCA TC-3'
	15R				5'-GAC TCC CAG AGC AAC TGT GCA TGT ACC ACC TAT C-3'
10	15F-2	65	10613	54241-64852	5'-TGA TAG GTG GTA CAT GCA CAG TTG CTC TGG-3'
	18R-2				5'-GCT AAC TAC CCA TTT TCC TCC CGC AAT TCC-3'
11	17F	61	4581	60922-65502	5'-GTG TAG AAC GTG CAG GAT TG-3'
	19R				5'-CAT TGT TAA GGA AAG TGG TGC-3'
12	18F	65	6897	64777-71673	5'-TGC AGA TGC TGA GTT TGT GTG TGA ACG GAC-3'
	20R				5'-CCT GGG ATT CTC TTG CTC GCT TTG GAC C-3'
13	ivs19F	65	7495	70154-77648	5'-TCC CAG TGA GGT GAA AAG CCG ATT GTT AAG TTC-3'
	21R				5'-CCC ATA GCA ACA GAT TTC TAG CCC CCT GAG G-3'
14	20F	61	9662	71518-81179	5'-ATA TGA CGT GTC TGC TCC AC-3'
	23R				5'-ACT GTG CTA CTC AAG CAC CA-3'
15	22F	63	8536	79560-88095	5'-AGC TGT GTG GTG CTT CTG TGG TGA AGG-3'
	24R				5'-AGA GCC AGC AAG ATC AGA TGG TCT ACA GGA C-3'

Table 3:

Identified RFLP Sites in *BRCA1*

Location	Primer Pair	Restriction Enzyme Site	Nucleotide Change
ivs 21	20F/23R	+ Sty I	C +390 T
ivs 3	3F/4R	- Ava I	ND
ivs 4	ivs 3F/7R	+ Sty I or Bgl II	ND*
ivs 8	8F/11R	- Eco RI	ND
ivs 12	11F/13R	- Pst I	ND
ivs 15	15F/18R	- Sma I	ND
ivs 15	15F/18R	+ Bgl II	ND
ivs 16 or 17	15F/18R	+ Eco RI	ND
ivs 19	18F/20R	- Ava I	ND
ivs 20	ivs 19F/21R	- Pst I	ND

*ND = not determined

Table 4: Loss of Heterozygosity Analysis within the BRCA1 region (17q21)

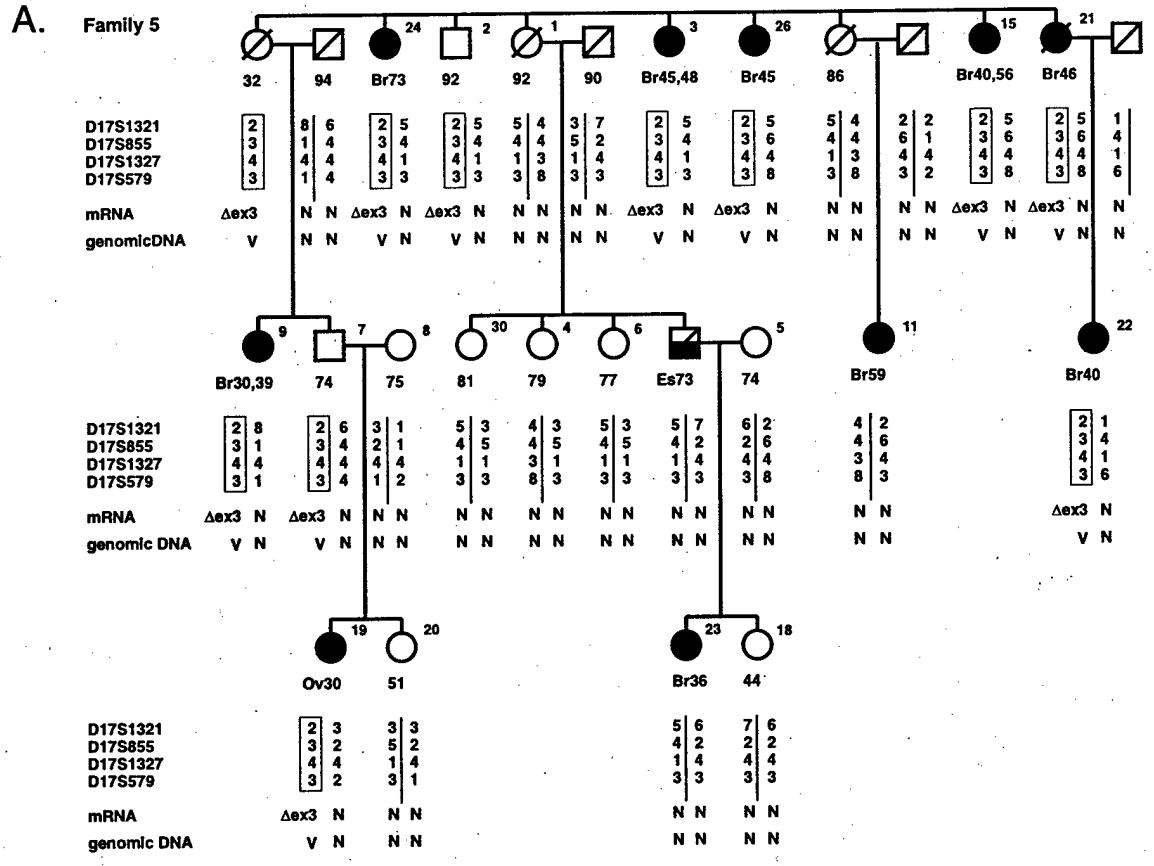
ID	Age	Type	Grade	Stage	% Tumor	D17S1320	D17S855	D17S1322	D17S1323	D17S1326	D17S1325
4003180F	42	invasive ductal	III	3		†●	†●	†●	○	†●	●
95:S1697	35	invasive ductal	III	3		●	●	●	●	●	●
4003218A	80	invasive ductal	III	2	80	●	●	●	●	●	●
148	47					~	●	●	●	●	●
11506F	41	invasive carcinoma	III	2		●	●	●	●	●	●
95-12-B003	65	invasive ductal	III	2	90	●	●	●	●	~	~
13633D	57	invasive ductal	III	2	50	~	●	●	●	●	○
96-05-A219	64	invasive ductal	I	2	30	○	●	●	●	●	●
95-08-B007	66	invasive ductal	I	2	100	●	●	●	●	●	~
94:S8766	35	invasive ductal	III	3		~	●	●	~	●	●
13207C	68				50	~	●	●	~	●	●
95:S5631	36	invasive ductal	III	2		●	●	●	~	●	~
96-05-F005	72	invasive ductal	III	2	30	○	●	~	●	~	~
96:S816	44	invasive ductal	I	2		~	●	~	●	~	~
96-01-C011R	49	invasive ductal	III	3		●	●	~	~	~	○
13917H	62	invasive ductal	III	2	80	~	●	~	~	~	●
95:S3559	38	invasive ductal	III	2		~	●	~	~	~	~
95:S7897	60	invasive ductal	I	2		○	●	~	~	~	~
95-06-A087	49	invasive ductal	I	2	80	●	●	~	~	●	~
95:S2994	66	invasive ductal	I	2	40	○	○	●	●	●	●
12864K	76	invasive mammary	I	3	60	○	○	○	○	●	●
95-09-C198	71	invasive mucinous	I	2	80	●	○	○	○	○	○
13949L	61	invasive ductal	III	3		●	○	~	~	●	●
11711B	50	invasive mammary	III	2		○	~	●	●	●	~
96-05-F002A	45	invasive ductal	III	3		●	~	●	●	~	~
12763K	45	invasive mammary	III	4		~	~	●	●	●	●
13331C	69	invasive ductal	III	2	70	●	~	●	~	●	~

Table 4: LOH at BRCA1 in 42 female patients with invasive breast cancer. ● indicates loss; ○ indicates retention of both alleles; ~ indicates uninformative; †● indicates gain of third allele in tumor. Histologic grade and TNM staging is indicated. D17S855, D17S1322, and D17S1323 are intragenic to BRCA1.

Table 4 (cont.): Loss of Heterozygosity Analysis within the BRCA1 region (17q21)

ID	*Age*	*Type*	*Grade*	*Stage*	*% Tumor*	*D17S1320*	*D17S855*	*D17S1322*	*D17S1323*	*D17S1326*	*D17S1325*
95:S10779	41	invasive apocrine	I	2		○	~	○	●	●	~
94:S7076	52	invasive ductal	I	4	70	●	~	~	~	~	●
13552C	72	invasive ductal	III	3	80	○	~	~	~	●	~
95:S12295	38	invasive ductal	I	2		~	●	~	~	●	~
95:S4617	54	invasive ductal	III	3		●	○	●	~	●	~
94:S7769	46	invasive ductal	III	2		~	●	~	~	~	~
95:S3156	69	invasive ductal		2		●	●	~	~	●	~
95:S2753	38	invasive ductal	III	2		~	●	●	~	~	~
95:S478	62	invasive ductal	III	3		○	○	○	~	~	●
97-03-A113B	37	invasive ductal	I	2		●	●	●	~	~	●
97:S3438	40	invasive ductal	III	1		~	●	●	●	●	~
97-05-C136R	63	invasive ductal	I	3		~	○	○	●	○	~
97-03-C285	69	invasive ductal	III	2	100	○	●	○	○	~	~
96-08-C061R	51	invasive ductal	III	2		○	○	~	~	~	●
97-03-C033G	46	invasive ductal	III	3	100	●	○	○	●	~	●

Table 4 (cont.): LOH at BRCA1 in 42 female patients with invasive breast cancer. ● indicates loss; ○ indicates retention of both alleles; ~ indicates uninformative. Histologic grade and TNM staging is indicated. D17S855, D17S1322, and D17S1323 are intragenic to BRCA1.



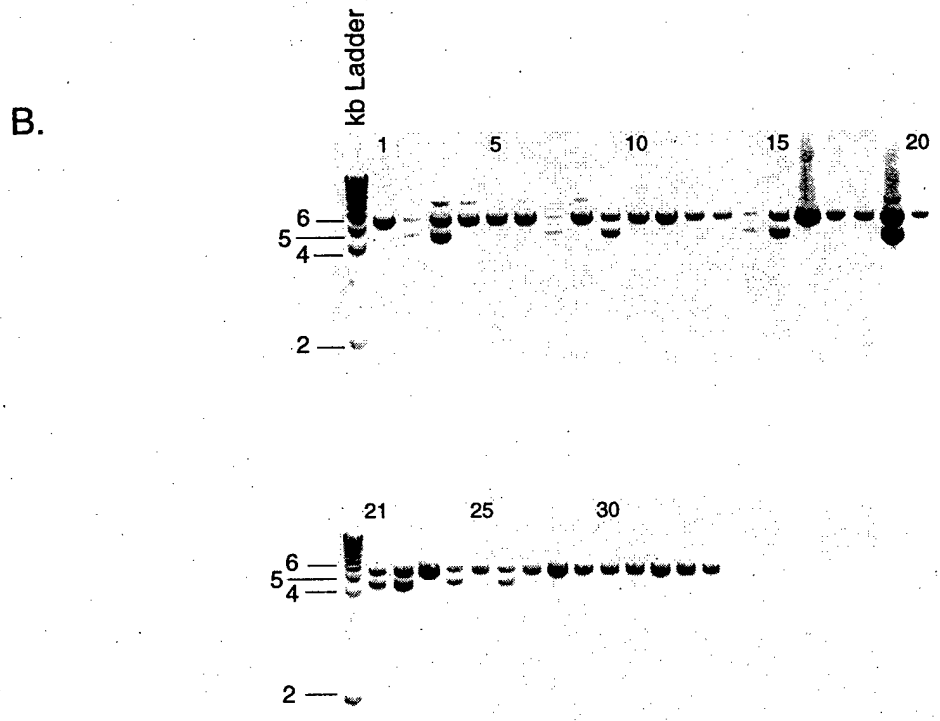


Figure 1: Pedigree and analysis of long range PCR products for Family 5

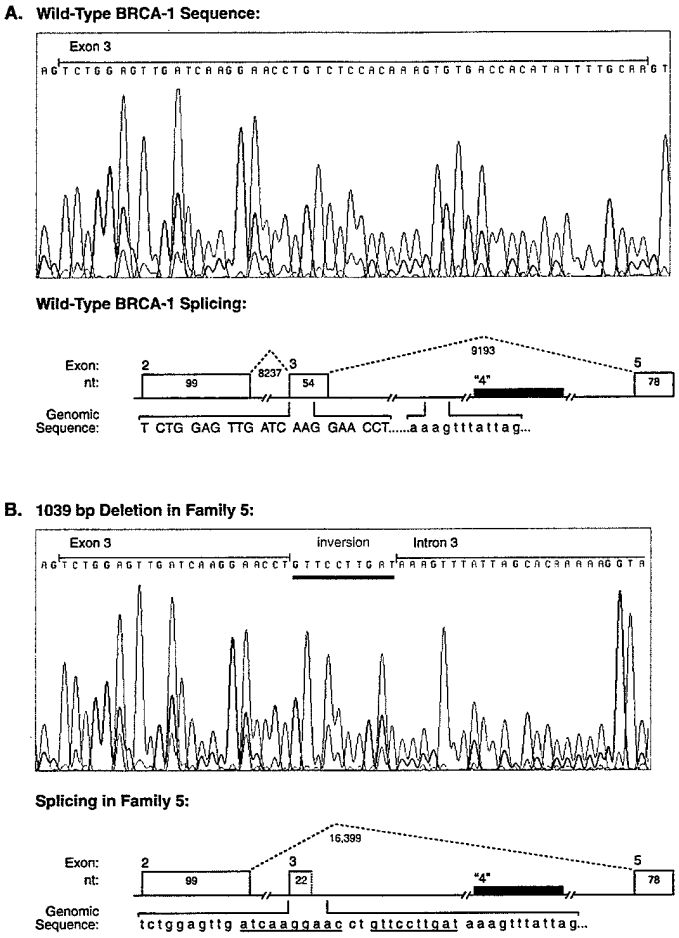


Figure 2: Exon skipping resulting in a truncated BRCA1 protein in Family 5

(A) Wild-type BRCA1 sequence and splicing of exon 3
(B) Breakpoint sequence of the 1039 bp deletion and effects on splicing in Family 5

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